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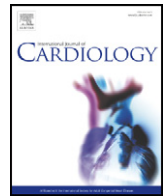
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Use of biomarkers to establish potential role and function of circulating microRNAs in acute heart failure☆



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ABSTRACT

Background: Circulating microRNAs (miRNAs) emerge as potential heart failure biomarkers. We aimed to identify associations between acute heart failure (AHF)-specific circulating miRNAs and well-known heart failure biomarkers.

Methods: Associations between 16 biomarkers predictive for 180 day mortality and the levels of 12 AHF-specific miRNAs were determined in 100 hospitalized AHF patients, at baseline and 48 hours. Patients were divided in 4 pre-defined groups, based on clinical parameters during hospitalization. Correlation analyses between miRNAs and biomarkers were performed and complemented by miRNA target prediction and pathway analysis.

Results: No significant correlations were found at hospital admission. However, after 48 hours, 7 miRNAs were significantly negatively correlated to biomarkers indicative for a worse clinical outcome in the patient group with the most unfavorable in-hospital course ($n = 21$): miR-16-5p was correlated to C-reactive protein ($R = -0.66$, p -value = 0.0027), miR-106a-5p to creatinine ($R = -0.68$, p -value = 0.002), miR-223-3p to growth differentiation factor 15 ($R = -0.69$, p -value = 0.0015), miR-652-3p to soluble ST-2 ($R = -0.77$, p -value < 0.001), miR-199a-3p to procalcitonin ($R = -0.72$, p -value < 0.001) and galectin-3 ($R = -0.73$, p -value < 0.001) and miR-18a-5p to procalcitonin ($R = -0.68$, p -value = 0.002). MiRNA target prediction and pathway analysis identified several pathways related to cardiac diseases, which could be linked to some of the miRNA-biomarker correlations.

Conclusions: The majority of correlations between circulating AHF-specific miRNAs were related to biomarkers predictive for a worse clinical outcome in a subgroup of worsening heart failure patients at 48 hours of hospitalization. The selective findings suggest a time-dependent effect of circulating miRNAs and highlight the susceptibility to individual patient characteristics influencing potential relations between miRNAs and biomarkers.

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Abbreviations: miRNA, microRNA; AHF, acute heart failure; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; VEGFR-1, vascular endothelial growth factor receptor 1; CRP, C-reactive protein; GDF-15, growth differentiation factor-15; sST-2, soluble ST-2; PCT, procalcitonin; TGF- β , transforming growth factor-beta.

☆ The authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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1. Introduction

Since the discovery of microRNAs (miRNAs) more than twenty years ago [1,2], the knowledge about their role in several disease processes has increased substantially. MiRNAs are small RNA particles (~22 nucleotides in length) with the ability to regulate gene expression at the post-transcriptional level [3,4]. This process mainly involves binding to complementary sequences of the messenger RNA (mRNA) after which it leads to translational repression or degradation of the mRNA [5]. MiRNAs are thought to play a role in a variety of pathophysiological mechanisms, including the development of heart failure [6].

Extracellular miRNAs are measurable in the circulation and are increasingly reported as potential diagnostic and prognostic biomarkers for multiple diseases [7]. Furthermore, owing to their stability, circulating miRNAs may be attractive new biomarkers in the cardiovascular field [8,9]. Recently, our group reported about a panel of 12 circulating miRNAs specifically associated with acute heart failure (AHF) [10]. These miRNA levels were found to be lower in patients with AHF compared to chronic heart failure patients, patients with an acute exacerbation of chronic obstructive pulmonary disease, and healthy controls. Further decreasing levels after 48 h of hospitalization in a subset of these miRNAs were predictive for 180 day mortality in patients with AHF. However, the meaning and role of these circulating miRNA levels in patients with AHF remain to be established.

Several other, protein based biomarkers in heart failure have been associated with different cardiac disease pathways such as angiogenesis, endothelial function, inflammation, cardiac stretch and remodeling, as well as to clinical outcome [11–13]. Early in-hospital worsening of heart failure is an important determinant for a worse prognosis [14–16]. We hypothesize that changes in AHF-specific miRNAs and other, protein based biomarkers between baseline and 48 h may indicate their role in heart failure related disease processes contributing to end organ damage and hence to a worse outcome. Therefore, by comparing the levels of biomarkers with miRNAs related to AHF, we aim to increase our understanding of the role and potential function of these specific miRNAs in the circulation. In the present study we study associations early during hospitalization between AHF-related circulating miRNAs and other novel and established biomarkers. We additionally use a bioinformatic approach to identify putative miRNA targets and enriched pathways to gain further insight into potentially related pathways and mechanisms.

2. Methods

2.1. Study population

A subset of 100 patients hospitalized for AHF from the Placebo-Controlled Randomized Study of the Selective A1 Adenosine Receptor Antagonist Rolofylline for Patients Hospitalized With Acute Decompensated Heart Failure and Volume Overload to Assess Treatment Effect on Congestion and Renal Function (PROTECT) was studied. The PROTECT study design and results have been described elsewhere [17,18]. The 24 healthy control subjects were derived from the Telosophy study, as previously reported [10,19]. The PROTECT and Telosophy study protocols conform to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval of the ethics committees of the participating centers. All patients gave informed consent.

2.2. Blood sample collection

Blood samples were collected at various time points during hospitalization, including baseline and 48 h. Plasma was collected from EDTA blood as reported before [17]. Briefly, plasma samples were shipped frozen to the central laboratory where central study measurements were performed. Samples were then stored in $-70^{\circ}\text{C}/-80^{\circ}\text{C}$ freezers and sent frozen to our center. Here, the samples were aliquoted and again stored in -80°C freezers.

2.3. Biomarker measurements

Laboratory values including albumin, alanine transaminase, aspartate transaminase, bicarbonate, blood urea nitrogen (BUN), chloride, creatinine, glucose, hemoglobin, platelet count, potassium, red blood cell count, sodium, total cholesterol, triglycerides, uric acid and white blood cell count were measured in a central laboratory (ICON Laboratories, Farmingdale, NY, USA). Frozen aliquots were sent to Alere™, (San Diego, CA), which were used for the protein based biomarker measurements. The biomarkers galectin-3, myeloperoxidase and neutrophil gelatinase-associated lipocalin were determined by means of sandwich enzyme-linked immunosorbent assays (ELISA) on a microtiter plate. Angiogenin and C-reactive protein (CRP) were measured using competitive ELISAs on a Luminex® platform. Sandwich ELISAs on a Luminex® platform were used for measuring D-dimer, endothelial cell-selective adhesion molecule, growth differentiation factor 15 (GDF-15), lymphotoxin beta receptor, mesothelin, neuropilin, N-terminal pro C-type natriuretic peptide, osteopontin, procalcitonin (PCT), pentraxin-3, periostin, polymeric immunoglobulin receptor, pro-adrenomedullin (proADM), prosaposin B, receptor for advanced glycation endproducts, soluble ST-2 (sST-2), syndecan-1, tumor necrosis factor alpha receptor 1 (TNFR-1), tumor necrosis factor receptor superfamily member, vascular endothelial growth receptor 1 (VEGFR-1) and WAP four-disulfide core domain protein HE4 (WAP-4C). The immunoassays for PCT, proADM, galectin-3 and ST2, developed by Alere, have not been standardized to the commercialized assays used in research or in clinical use and the extent to which each Alere assay correlates with the commercial assay is not fully characterized. Details about the biomarker assays including cut-off values, detection limits and inter assay coefficients of variation were published elsewhere [20].

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

MiRNA isolation and profiling were conducted in the same laboratory with standard procedures and commercial kits and assays as detailed in the recent paper of Ovchinnikova et al. [10] and the Supplementary Material. Once the samples were thawed, the miRNA isolation and PCRs were directly performed, avoiding additional freeze/thaw cycles. The miRCURY RNA isolation kit for bodyfluids (Exiqon, Vedbaek, Denmark) was used to isolate RNA from 200 μl of plasma. Using the Exiqon Serum/Plasma Focus microRNA PCR panel, the expression of 375 miRNAs was measured in 10 AHF patients. Fifteen miRNAs which exhibited a statistically significant difference (with a Bonferroni corrected p-value threshold of ≤ 0.00022) and a 4-fold change in the AHF samples compared to the control samples were selected and analyzed in an extended cohort of 100 AHF patients. For this, a customized panel containing the 15 miRNAs of interest was used (Exiqon). The reference genes miR-30a-5p and miR-194-5p were selected and remained constant in the patient and control samples. Expression levels were normalized against these reference genes and the comparative delta-delta-CT method was used to calculate relative expression levels (GenEx Professional software, MultiD Analyses, Sweden). Out of these 15 miRNAs, 12 miRNAs (let-7i-5p, miR-16-5p, miR-18a-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-106a-5p, miR-199a-3p, miR-223-3p, miR-423-3p, miR-423-5p and miR-652-3p) were validated in an independent AHF cohort and were used for the analyses in this study [10].

2.5. Statistical analyses

MiRNA expression data was handled using GenEx Professional software, MultiD Analyses, Sweden. All other statistical analyses were conducted using R: A Language and Environment for Statistical Computing, version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria). Receiver operating characteristic (ROC) analyses were performed to obtain the area under the ROC curve, using the R package pROC.

Univariable Cox proportional hazards regression analysis was performed to determine the predictive value of the biomarkers for 180 day mortality, including Harrell's C-index calculation. Correlation analyses were performed using the Spearman Rank test from the R package Hmisc. Hierarchical clustering of correlation coefficients and illustrating them in heatmaps were performed using the R package gplots. Principle component analysis (PCA) was conducted for the miRNAs and biomarkers in all clinical groups at baseline and 48 h using the R packages devtools and ggbiplot. The purpose of this method, as previously used by others [21], was to correct for multiple testing in our correlation analyses based on the number of components explaining 95% of the variance of the miRNAs and biomarkers in all clinical groups at baseline and 48 h, leading to a corrected p-value threshold of 0.002941 for baseline and 0.002778 for 48 h (Supplementary Table 1). P-values of <0.05 were deemed statistically significant for all other analyses. Target prediction of the miRNAs was performed by means of the DNA Intelligent Analysis (DIANA)-micro T-CDS (v.5.0) prediction algorithm [22] after which pathway analysis was conducted with DIANA-miRPath (v.2.0) [23]. The micro T-CDS threshold score for predicted targets was set at 0.8 and the p-value threshold at $p < 0.05$. Correcting for multiple testing was taken into account using False Discovery Rate (FDR) correction.

3. Results

3.1. Diagnostic accuracy of the selected miRNAs

In a previous publication of our group we identified a set of circulating miRNAs associated with AHF [10]. To confirm the discriminating properties of the selected miRNAs between the investigated AHF cohort and a group of 24 previously described healthy control patients [10], we performed ROC analyses. All 12 miRNAs showed high area under the ROC curve (AUC) values within a range of 0.82–0.97 (as presented in Supplementary Table 2).

3.2. Selection of biomarkers

We selected from the total PROTECT dataset ($n = 2033$) 16 out of 43 established and novel biomarkers significantly predictive for 180 day all-cause mortality at 48 h with a C-index of ≥ 0.60 (Table 1). In the

majority of cases, higher levels of biomarkers were indicative for a worse clinical outcome. However, for albumin, total cholesterol, sodium and triglycerides, lower levels were predictive for 180 day all-cause mortality.

3.3. Selection of patient groups

The subpopulation of 100 AHF patients was comparable to the total PROTECT population (Supplementary Table 3). Patients were divided into 4 groups based on clinical characteristics and the trichotomous endpoint of the PROTECT study [18] (Treatment Failure, Success and Unchanged), which reflects the in-hospital clinical course of the patients. To further distinguish the patients with the worst in-hospital course in the Failure group, additional criteria divided the Failure patients in *failure-intermediate* and *failure-worsening* patients. The group criteria have been schematically depicted in Fig. 1. The baseline characteristics (Table 2) show that groups did not significantly differ except for a worse renal function and higher numbers of prior beta-blocker use in the *failure* groups and small differences in the New York Heart Association (NYHA) classification. In contrast, the clinical in-hospital characteristics and outcome parameters show a clear shift from patients in the *improving* and *no change* groups to patients belonging to the *failure* groups and especially the *worsening* group, with higher mortality rates in the *failure* groups.

3.4. Correlation between miRNAs and biomarkers

Correlation analyses were performed for the 12 miRNAs and 16 selected biomarkers at baseline and 48 h. The p-value threshold to reach significance was adjusted to correct for multiple testing by dividing 0.05 by the components which explained 95% of the variance per time point, resulting from the PCA. For baseline, a total of 17 principal components explained 95% of the cumulative proportion of variance and consequently, the adjusted p-value threshold was set as $0.05/17 = 0.002941$. For 48 h, a total of 18 principal components resulted in an adjusted p-value threshold of $0.05/18 = 0.002778$ (Supplementary Table 1). At baseline level, no significant correlations were found between the circulating miRNAs and biomarkers. Correlation analyses at 48 h of hospitalization showed a clear gradual trend of more negative correlations from the *improving* group to the *failure-worsening* group (Fig. 2). Overall, 9 significant negative correlations were detected. One significant negative correlation in the *improving* group between miR-30e-5p and VEGFR-1 ($R = -0.59$, p-value = 0.002) and 1 between let-7i-5p and triglycerides ($R = -0.51$, p-value = 0.0027) in the *failure-intermediate* group. Seven out of 9 significant negative correlations were observed in the *failure-worsening* group between miR-16-5p and CRP ($R = -0.66$, p-value = 0.0027), miR-106a-5p and creatinine ($R = -0.68$, p-value = 0.002), miR-223-3p and GDF-15 ($R = -0.69$, p-value = 0.0015), miR-652-3p and sST-2 ($R = -0.77$, p-value < 0.001), miR-199a-3p and PCT ($R = -0.72$, p-value < 0.001), miR-18a-5p and PCT ($R = -0.68$, p-value = 0.002) and miR-199a-3p and galectin-3 ($R = -0.73$, p-value < 0.001). A complete list of all correlation results are depicted in Supplementary Table 4.

3.5. In silico miRNA target prediction and pathway enrichment analysis

Computational bioinformatic tools were used to identify putative miRNA targets. Targets were predicted by means of DIANA-micro T-CDS (v.5.0). Of these predicted targets, 4 target genes were identified encoding for 4 of our selected biomarkers. MiR-30e-5p and miR-106-5p were found to target FLT1, encoding for VEGFR-1 (predicted by DIANA microT-CDS, TargetScan and MiRanda), miR-199a-3p targets IL1RL1, encoding for sST-2 (predicted by DIANA microT-CDS and MiRanda) and miR-27a-3p has ALB as putative target, encoding for albumin (predicted by DIANA microT-CDS and MiRanda). Interestingly, miR-30e-5p and VEGFR-1 were also significantly negatively correlated

Table 1
Cox proportional hazard regression analysis for 180 day all-cause mortality.

Biomarker	Hazard ratio (95% CI)	P-value	C-index
BUN	1.8 (1.6–2.0)	<0.001	0.67
sST-2	1.7 (1.5–1.9)	<0.001	0.65
WAP-4C	1.6 (1.4–1.9)	<0.001	0.63
VEGFR-1	1.5 (1.4–1.7)	<0.001	0.63
Total cholesterol	0.6 (0.5–0.7)	<0.001	0.63
CRP	1.5 (1.4–1.8)	<0.001	0.62
ProADM	1.7 (1.4–1.9)	<0.001	0.62
Procalcitonin	1.5 (1.3–1.6)	<0.001	0.62
Galectin-3	1.5 (1.3–1.7)	<0.001	0.61
TNFR-1	1.5 (1.3–1.6)	<0.001	0.61
Albumin	0.7 (0.6–0.8)	<0.001	0.61
Syndecan-1	1.4 (1.2–1.6)	<0.001	0.60
GDF-15	1.5 (1.3–1.7)	<0.001	0.60
Sodium	0.7 (0.7–0.8)	<0.001	0.60
Triglycerides	0.7 (0.6–0.8)	<0.001	0.60
Creatinine	1.4 (1.3–1.6)	<0.001	0.60

Univariable Cox regression analysis at 48 h of hospitalization. Biomarkers with a C-index of ≥ 0.60 are depicted with the Hazard Ratio (95% CI) and p-value. Hazard ratio should be interpreted per standard deviation. All biomarkers except for albumin, proADM, sodium and total cholesterol were log-transformed. Performance of the model was calculated with the concordance index (C-index). BUN indicates blood urea nitrogen; sST-2, soluble ST-2; WAP-4C, WAP four-disulfide core domain protein HE4; VEGFR-1, vascular endothelial growth factor receptor 1; CRP, C-reactive protein; proADM, pro-adrenomedullin; TNFR-1, tumor necrosis factor alpha receptor 1 and GDF-15, growth differentiation factor 15.

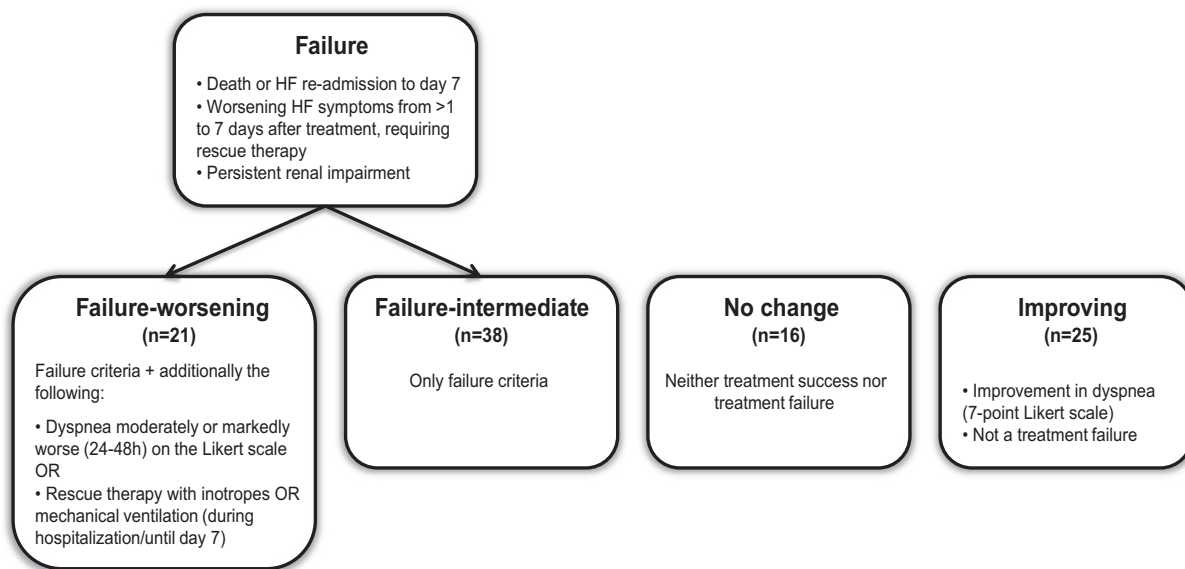


Fig. 1. Patient grouping according to PROTECT primary endpoint definitions. Patients ($n = 100$) were divided in 4 groups based on the trichotomous endpoint of the PROTECT study; Treatment Failure, Unchanged (=no change) or Success (=improving). The patients in the Failure group were further divided in *failure-worsening* and *failure-intermediate* based on additional clinical criteria for the *failure-worsening* group.

in our dataset in the *improving* group (Fig. 2). Pathway analysis on the predicted targets using DIANA-miRPath (v.2.0) led to the identification of 47 significant KEGG pathways in which the predicted miRNA targets were enriched (Fig. 3, Supplementary Table 5). Multiple miRNAs and corresponding predicted target genes were involved in top-enriched pathways which have previously been implicated in cardiac disease and heart failure, including the PI3K-Akt signaling pathway ($p = 1.80e^{-12}$), ErbB signaling pathway ($p = 6.22e^{-12}$), transforming growth factor- β (TGF- β) signaling pathway ($p = 6.87e^{-11}$) and ubiquitin mediated proteolysis ($p = 1.45e^{-10}$). In a set of 4274 cardiovascular related genes extracted from the Cardiovascular Gene Ontology Annotation list (<http://www.ebi.ac.uk/QuickGO/GProteinSet?id=BHF-UCL>), 1178 (23%) of the AHF-associated miRNA targets, as predicted by DIANA-micro-T-CDS, overlapped with the cardiovascular related genes.

4. Discussion

Increasing interest in circulating miRNAs has led to several questions regarding their role in the circulation, origin and reflection of various disease processes. With the present study, we aimed to gain insight in the role and potential function of circulating miRNAs in AHF patients with the help of other well-known circulating biomarkers. First, we showed that correlations between biomarkers and miRNAs not only depend on the time of measurement, but on the disease state of the patient as well. Specifically in those patients with the worst clinical course during hospitalization, which were characterized by a worsening renal function, the need of rescue therapy, worsening dyspnea symptoms at 24 to 48 h and a poor outcome, we found several significant negative correlations. The negative correlations reflect an inverse relationship in which biomarker levels increase and miRNA levels decrease and vice versa. These results are in line with the previously found association of this panel of decreasing miRNAs with the most acute state of heart failure [10], which are strengthened by this corresponding biomarker profile. In contrast, no significant correlations were found at time of admission and only 1 in both the *failure-intermediate* and *improving* group at 48 h of hospitalization, suggesting that variation in clinical characteristics, medication use and the time point of measurement during hospitalization may be of great influence. This may be valuable

information for future research regarding circulating miRNAs and biomarkers and brings additional challenges to light.

Six of the 7 biomarkers with significant correlations with 7 of the AHF-associated miRNAs are known to play a role in important cardiac disease processes such as inflammation, cardiac remodeling/fibrosis and angiogenesis [24]. GDF-15 and CRP are inflammatory markers and also the level of PCT rises in response to pro-inflammatory stimuli [25, 26]. Increased levels of sST-2, a novel biomarker of cardiac stress, reflect the severity of adverse cardiac remodeling and tissue fibrosis in response to myocardial infarction, acute coronary syndrome or worsening heart failure [27]. Galectin-3 is involved in multiple biological processes and has been shown to play a role in inflammation, fibrosis and heart failure [28–30].

The correlation identified in the *failure-worsening* group between CRP and miR-16-5p is supported by the study of Castro-Villegas et al. [31] MiR-16-5p was found to be significantly upregulated following therapy with anti-TNF α /disease-modifying anti-rheumatic drugs in patients with rheumatoid arthritis. Only responder patients showed an increase in miR-16-5p and 5 other miRNAs after therapy which was paralleled by the reduction of CRP. Therefore, as this previous study and our data demonstrate, miR-16-5p might be co-regulated with the inflammation factor CRP.

Another observed correlation between galectin-3 and miR-199a-3p in the *failure-worsening* group might be explained by the relevance of cardiac remodeling and fibrosis in heart failure. Galectin-3 expression is increased in cases of liver-, renal- and cardiac fibrosis as well as idiopathic pulmonary fibrosis [29,32–34]. MiR-199a was also associated with heart failure [35] and fibrotic processes in the lung and liver [36–38]. In a recent study of Yang et al. miR-199a-5p was significantly increased in serums of idiopathic pulmonary fibrosis patients [39]. These results suggest a potential interaction between miR-199a and fibrotic processes which might also be important in heart failure.

Furthermore, miR-652-3p was found to be correlated with sST-2 in the *failure-worsening* group. Decreased serum levels of miR-652 in patients with chronic liver disease suggest a putative role of this miRNA in the mediation of fibrogenic and inflammatory processes which may explain the observed correlation with sST-2, a marker of fibrosis and inflammation elevated in patients under cardiac stress [40]. Interestingly, low levels of circulating miR-652-3p were predictive for a heart failure admission within 5 years in post-acute coronary syndrome patients

Table 2
Baseline- and outcome characteristics of the 4 AHF patient groups.

Characteristics	Failure		No change	Improving	P-trend
	Worsening (n = 21)	Intermediate (n = 38)	(n = 16)	(n = 25)	
Demographics					
Sex, % male (n)	57.1 (12)	42.1 (16)	75 (12)	40 (10)	0.644
Age (years)	63.1 ± 12.2	73.5 ± 9.2	67.1 ± 11.8	68 ± 11.4	0.413
Systolic Blood Pressure (mmHg)	110.3 ± 13.8	123.4 ± 18.4	120.3 ± 14.4	120.5 ± 17.4	0.082
Diastolic Blood Pressure (mmHg)	66.8 ± 10.7	72.1 ± 11	72.5 ± 10.9	73 ± 13.8	0.082
Heart Rate (beats/min)	75.3 ± 8.8	79.7 ± 16.3	74.8 ± 17	82.4 ± 17.8	0.262
Rolofylline Administration, % (n)	52.4 (11)	68.4 (26)	62.5 (10)	64 (16)	0.611
Clinical profile					
Orthopnea, % (n)	95.2 (20)	94.7 (36)	87.5 (14)	100 (25)	0.596
Rales, % (n)	38.1 (8)	57.9 (22)	75 (12)	60 (15)	0.119
Edema, % (n)	76.2 (16)	71.1 (27)	62.5 (10)	60 (15)	0.190
Creatinine (mg/dL)	2 [1.6–2.2]	1.4 [1.2–1.9]	1.3 [1.2–1.7]	1.2 [1.1–1.4]	<0.001
Creatinine Clearance (ml/min)	33.4 [25.9–40.1]	42.2 [35.1–57.5]	53.4 [38.9–64.4]	57.8 [44.6–66.7]	<0.001
Blood Urea Nitrogen (mg/dL)	50 [36–83]	30 [26–45.8]	28.5 [24.8–40]	25 [22–32]	<0.001
Medical history, % (n)					
Heart failure	100 (21)	94.7 (36)	93.8 (15)	96 (24)	0.571
Hypertension	76.2 (16)	84.2 (32)	75 (12)	92 (23)	0.252
Diabetes Mellitus	42.9 (9)	55.3 (21)	31.2 (5)	36 (9)	0.280
Hypercholesterolemia	61.9 (13)	47.4 (18)	37.5 (6)	44 (11)	0.221
Ischemic Heart Disease	76.2 (16)	73 (27)	81.2 (13)	68 (17)	0.646
Atrial Fibrillation	61.9 (13)	63.2 (24)	50 (8)	52 (13)	0.339
Stroke	14.3 (3)	10.5 (4)	6.2 (1)	4 (1)	0.190
COPD	19 (4)	21.1 (8)	6.2 (1)	8 (2)	0.136
NYHA Class					0.021
2	19 (4)	13.2 (5)	12.5 (2)	16 (4)	
3	52.4 (11)	52.6 (20)	50 (8)	60 (15)	
4	28.6 (6)	28.9 (11)	31.2 (5)	20 (5)	
Hospitalized for heart failure in the past year	76.2 (16)	68.4 (26)	62.5 (10)	56 (14)	0.132
Prior medication use, % (n)					
ACE inhibitors or ARB	57.1 (12)	68.4 (26)	68.8 (11)	76 (19)	0.204
Beta Blockers	90.5 (19)	78.9 (30)	75 (12)	64 (16)	0.034
Mineralocorticoid Receptor Antagonists	61.9 (13)	50 (19)	56.2 (9)	36 (9)	0.116
Clinical outcomes					
Diuretic Response ^a (kg)	−0.1 ± 0.4	−0.3 ± 0.4	−0.4 ± 0.5	−0.6 ± 0.8	0.004
Weight change baseline - day 4 (kg)	−0.3 ± 3.7	−2.3 ± 2.7	−2.5 ± 2.3	−3.3 ± 3.3	0.005
Total diuretic dose, baseline - 48 h (mg)	680 [249–960]	309.9 [152.5–532.5]	240 [152.5–305]	235.6 [160–280]	<0.001
Inotropics, % (n)	85.7 (18)	0 (0)	0 (0)	0 (0)	<0.001
Moderately or marked worsening of dyspnea at 24 h and/or 48 h, % (n)	28.6 (6)	0 (0)	0 (0)	0 (0)	0.001
Mechanical Ventilation, % (n)	9.5 (2)	0 (0)	0 (0)	0 (0)	0.055
WRF, day 7, % (n)	38.1 (8)	42.1 (16)	0 (0)	12 (3)	0.003
Treatment failure due to death, % (n)	4.8 (1)	0 (0)	0 (0)	0 (0)	0.177
Treatment failure due to Worsening Heart Failure, % (n)	95.2 (20)	78.9 (30)	0 (0)	0 (0)	<0.001
Treatment failure due to WRF, % (n)	28.6 (6)	34.2 (13)	0 (0)	0 (0)	0.001
Mortality and rehospitalization, % (n)					
180-day all-cause Mortality	42.9 (9)	23.7 (9)	12.5 (2)	12 (3)	0.013
60-day all-cause Mortality	33.3 (7)	7.9 (3)	0 (0)	4 (1)	0.003
60-day Heart Failure Rehospitalization	0 (0)	13.2 (5)	25 (4)	20 (5)	0.040
60-day Rehospitalisation	14.3 (3)	28.9 (11)	37.5 (6)	24 (6)	0.486

Characteristics of the 4 groups; *failure-worsening*, *failure-intermediate*, *no change* and *improving*. Values are depicted as mean ± SD or median and interquartile range. COPD indicates chronic obstructive pulmonary disease; NYHA, New York Heart Association; ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker and WRF, worsening renal function.

^a Diuretic Response: kilogram weight loss on day 4 per 40 mg of Lasix through day 3.

[41], in consensus with our previous study in which we report on the predictive value miR-652-3p for mortality in heart failure patients [10].

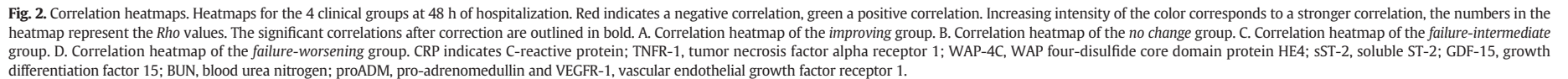
We found miR-106a-5p to be negatively correlated to creatinine, which suggests a potential role in renal function. In another study by our group, we previously demonstrated that low levels of this miRNA were related to patients with worsening of renal function and higher levels of NGAL [42]. This is in conjunction with the presently found association of this miRNA with creatinine in specifically those patients with the least favorable in-hospital course.

The other significant negative correlations between miR-199a-3p and PCT, miR-18a-5p and PCT, and miR-223-3p and GDF-15 all suggest a potential relation with inflammatory processes, although specific relations between these miRNAs and biomarkers have not been described before. Similarly, no literature was found supporting the association between let-7i-5p and triglycerides in the *failure-intermediate*

group, although involvement of the let-7 family in lipid metabolism has been previously reported [43,44].

We hypothesize that not all potential associations between the selected miRNAs and biomarkers were reflected by the results of the performed correlation analyses. A clear shift was observed towards more negative correlations between miRNAs and biomarkers in the *failure-worsening* group at 48 h, but the clinically complex nature of AHF in combination with the relatively low adjusted p-value threshold might not allow for the statistical significant detection of all relevant correlations at a given time point within the limited number of patients.

MiRNAs fine tune cellular responses by regulating a large number of targets in various pathological and physiological conditions. MiRNAs can alter the expression of signaling molecules within a particular pathway and evidence accumulates that they could also act as modulators between multiple pathways [45]. In addition to our attempt to use known biomarkers to position the AHF-related miRNAs in relevant



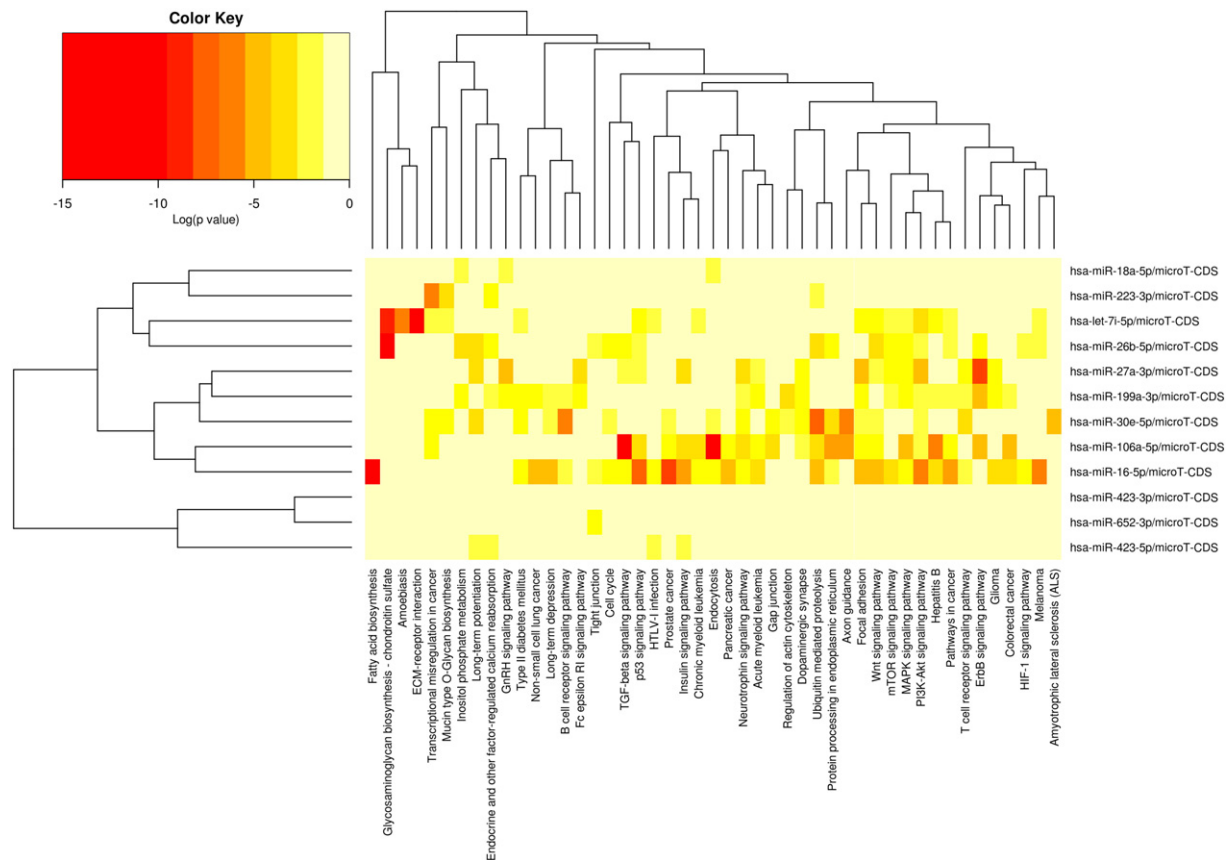


Fig. 3. Pathway analysis heatmap. Significant pathways predicted by DIANA-miRPath (v.2.0) are depicted on the X-axis and miRNAs on the Y-axis. The color code represents the log(p-value), with the most significant predicted miRNA-pathway interactions in red.

heart failure disease processes, we performed miRNA target prediction followed by pathway analysis.

Target prediction identified FLT1 encoding for VEGFR-1 to be targeted by miR-30e-5p and miR-106a-5p by 3 independent prediction programs. In TarBase, FLT1 is listed as a immunoprecipitation (IP) validated target of miR-106-5p [46]. VEGFA and VEGFC are IP validated targets of miR-30e-5p, which are closely linked to VEGFR-1 [47–49]. As reviewed recently, soluble FLT1 plays an anti-angiogenic role whereas VEGF acts as pro-angiogenic factor in peripartum cardiomyopathy (PPCM) [50]. Increased FLT1 results in declined VEGF levels, in which miRNAs might play a regulatory role. Insufficient cardiac expression of VEGF has been suggested to lead to endothelium dysfunction and heart failure development due to impaired blood flow [51]. These findings strengthen the validity of the detected correlation between VEGFR-1 and miR-30e-5p. Although there is no evidence in heart disease that would support the detected correlation between miR-30e and VEGFR-1 in the *improving* group, in a breast cancer study, the suppression of another miR-30 family member, miR-30b, was associated with overexpression of VEGF genes [52].

For the biomarkers sST-2 and albumin predicted to be targeted by miR-199a-3p and miR-27a-3p respectively, no supportive evidence of a functional interaction could be found in the current literature.

Target prediction via DIANA-micro T-CDS resulted in a list of predicted genes based on complementary 3' untranslated regions and coding sequences of the mRNA. Pathway analysis on the predicted targets using DIANA-miRPath (v.2.0) led to 47 significantly enriched KEGG pathways (Supplementary Table 5). PI3K-Akt, ErbB, TGF- β and focal adhesion signaling pathways as well as the ubiquitin mediated proteolysis ranked among the top 10 enriched pathways. All of these pathways are described to play a role in the development of heart failure. The PI3K-Akt pathway regulates cardiomyocyte size, survival, angiogenesis and

inflammation in both physiological and pathological cardiac hypertrophy [53]. It has also been reported that Akt and mTOR contribute to angiogenesis by increasing the expression of VEGF and angiopoietin-2 [53]. TGF- β plays a central role in induction of fibrosis and also accelerates production of extracellular matrix (ECM) proteins [54]. Focal adhesion signaling pathways have been described as contributing factors to fibrosis and cardiomyocyte hypertrophy [55].

The identification of top ranking signaling pathways involved in cardiac remodeling, angiogenesis and inflammation coincides with the clustering of the biomarkers that were found to be significantly associated with 7 of the 12 AHF-related miRNAs.

4.1. Study limitations

Although we have identified interesting correlations between biomarkers and miRNAs in patients with AHF, factors such as time dependent miRNA and biomarker expression and clinical status challenged the approach to use known functions of biomarkers to mechanistically position the AHF-related miRNAs. In this regard, concomitant diseases, medication and volume status contribute to the complex phenotype of the AHF population and may have an influence on circulating biomarkers and miRNAs. The study is further limited by the small sample size of the subgroups and the lack of comparable healthy control patients. More information about the relation of circulating miRNAs and biomarkers might be found in chronic heart failure patients, resembling a more stable condition than patients hospitalized for AHF. Furthermore, the biological function of miRNAs in the circulation remains elusive and whether these miRNAs can exert regulative effects on pathways involved in heart failure, needs further investigation. Since heart failure is a heterogeneous syndrome mostly affecting patients with several comorbidities, a certain overlap in underlying mechanisms

of other diseases is not uncommon and therefore the identified pathways may also be of importance in related comorbidities. Due to the robustness of the results from our previous study [10] and the herein described associations with biomarkers previously related to heart failure, we are confident about the reported correlations and their involvement in AHF. Nonetheless, we emphasize on the need to experimentally validate our results, as these results can only be regarded as hypothesis generating. Experimental studies in a controlled environment (e.g. cultured cardiomyocytes) with overexpressed or knock-down miRNAs are more likely to yield pathway and disease-related data.

However, to our knowledge, this is the first study investigating the relation of circulating miRNAs in patients with AHF with a large panel of biomarkers, in contrast with most studies which are mostly limited to (NT-pro)BNP and troponin.

5. Conclusions

We found several associations between miRNAs and other, established and novel biomarkers corresponding to a worse prognosis in AHF patients with an unfavorable in-hospital course, at 48 h of hospitalization. Several miRNA-biomarker correlation pairs were identified in these worsening heart failure patients which could be linked to predicted targets and pathways related to cardiac disease, such as cardiac remodeling/fibrosis, inflammation and angiogenesis.

Funding and conflicts of interest

This study was supported by a Grant from the Dutch Heart Foundation: Approaching Heart Failure By Translational Research Of RNA Mechanisms (ARENA). The PROTECT trial was supported by NovaCardia, a subsidiary of Merck. E.L.V., D.S., Y.H., P.V.D.H. and M.A.F.K. have nothing to disclose. J.R.T. has received research funds and consulting fees from Actelion, Amgen, Cytokinetics, Merck, and Novartis. C.M.O.C. is a consultant to Merck. M.M. has received consulting incomes from Abbot Vascular, Bayer, Novartis and Servier. G.C. and B.D. are employees of Momentum Research Inc., which was contracted to perform work on the project by Merck & Co, Inc. D.M.B. is an employee of Merck. J.G.C. was on the Steering Committee of the study, served on the Advisory Board for MSD, and received payments for both. M.M.G. has received institutional research support and served on a scientific Advisory Board for Merck. P.P. has received honoraria from Merck. D.J.V.V. has received Board Membership fees from Amgen, Biocontrol, Johnson & Johnson, Novartis, Sorbent and Vifor. A.A.V. was a member of the Steering Committee of PROTECT. E.B. is a co-founder and member of the scientific advisory board of InterRNA Technologies B.V., which develops miRNA therapeutics for cancer. A.A.V., E.B., E.S.O. and P.V.D.M. are patent holders of the circulating miRNAs in this manuscript. No other conflicts were reported.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ijcard.2016.09.010>.

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